the substrate exhibited linear detector response. RNA detection by UV absorbance was found to be a limiting factor in the Michaelis–Menten analysis.

Characterization of pre-tRNA maturation by RNase using capillary gel electrophoresis A CGE-based technique has been developed in order to characterize the reaction kinetics and mechanism for maturation of a set of pre-tRNA^{fMet} mutants. At all steps of the study of RNase P, including the preparation of the pre-tRNA (quality), the kinetic analysis and the control and yield of the purification steps, CGE was found appropriate and reliable.

Analysis of a ribonuclease H digestion of N3′–P5′ phosphoramidate–RNA duplexes by capillary gel electrophoresis The activity of a ribonuclease H (RNase H)-mediated RNA hydrolysis of duplexes formed by oligodeoxyribonucleotides or their analogue, N3′–P5′ phosphoramidates and complementary RNA strands, have been investigated. The enzymatic assay conditions were carefully optimized enabling sampling directly from the reaction mixture. CGE electropherograms revealed that RNA–N3′–P5′ phosphoramidates duplexes remained intact and therefore did not appear to be a substrate for RNase H.

Conclusion

Today, CE of nucleic acids has become an important analytical technique for biochemists and molecular biologists and the scientific studies described here clearly illustrate the applicability of CE in the analysis of RNA. Through efficient separations of RNA molecules ranging from a few bases to several kilobases, the specific and sensitive detection of RNA sequences and the study of RNA reaction kinetics, scientists have taken advantage of the prominent characteristics of CE. Compared to the analysis of DNA, additional challenges exist in the analysis of RNA, challenges mainly related to RNA stability and conformation. However, efforts should be made to overcome problems related to inefficient separation, identification and detection of RNA in CE. Extended insight into these phenomena will realize the inherent potential of CE for a diversity of RNA analyses.

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SELECTIVITY OF IMPRINTED POLYMERS: AFFINITY SEPARATION

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Ever since the discovery of antibodies and receptors, and their remarkable selectivities for almost any given chemical structure, scientists have been intrigued by the quest of mimicking their properties in synthetic or semisynthetic systems. A material carrying a selective preference for one ligand in comparison with other structurally similar compounds would be of outstanding use in a wide variety of situations, extending from molecular transportation, via analysis, to catalysis and synthesis. A multitude of sophisticated approaches have also been developed over the years, with the objective of controlling ligand binding to an artificial receptor.

Molecular imprinting technology (MIT) is an emerging concept that meets the objective of creating substrate selective materials in a fairly simple, yet efficient, manner. This technique is based on the formation of binding sites, or 'imprints', in a macromolecular matrix, or other suitable molecular support, by a molecular casting procedure. The ligand of interest is thus acting as an active guide, positioning the formation of the binding site, and the outcome is a two- or three-dimensional network embracing the template. Using dynamic interactions between the ligand (or its substitute) and selected building blocks, materials carrying a memory of the ligand structure can be formed. Once the formation of the network is established, the ligand can be removed, thus exposing the binding sites, which are subsequently accessible for repeated binding. A more general description of the molecular imprinting concept is presented elsewhere in this Encyclopedia.

The ligand affinities that can be obtained by this concept are quite remarkable and are often very similar to what can be achieved by the natural systems they are mimicking. Binding constants in the nanomolar range have been reported. Likewise, the selectivities they display are conspicuous, such that very small differences in ligand structure can be distinguished. Thus, the dynamic arrangement of interacting groups in the binding site, moulded in place by the imprinting process, can result in very specific interactions between the imprinted material and the targeted ligand. In this section, the selectivity of molecular imprinted polymers and other matrices will be discussed, focusing on their use in affinity separation.

Molecularly Imprinted Materials

Of all areas where molecularly imprinted materials have found applications, such as diagnostic assays, drug delivery, sensor technology, and catalytic protocols, by far the most explored field is their use in separation science. Such materials have been developed for use in liquid chromatography (molecular imprinting chromatography, MIC), solid-phase extraction (SPE), capillary electrophoresis, membrane technology and library screening protocols. Especially, chiral discrimination has been heavily investigated, and a large number of impressive chiral separations have been performed. More detailed overviews with respect to chiral separation, as well as to SPE, by these materials can be found elsewhere in this Encyclopedia.

Affinity Separation

As is the case with all types of affinity-based separation techniques, imprint-based protocols can be considered as a mixed-type separation process. The recognition between the analytes and the matrix relies on both affinity-type interactions, exerted by the ensemble of interactions between the analyte and the matrix in the recognition site, and less selective processes, such as general ion exchange, metal coordination and hydrophobic interactions. Although this mixed-type separation can sometimes be advantageous in as much as it offers a means for the analytical chemist to control the retention, most often it is a drawback, and attempts have been made to mask unwanted recognition contributions. For example, the nonselective ion exchange contribution of charged moieties in the matrix can be chemically blocked, resulting in a material where only the charged groups in the sites are active (**Figure 1**).

The phenomenon of nonspecific binding can sometimes be difficult to distinguish from the affinity process, since a comparison between a material that has been imprinted to a nonimprinted blank can result in differences for compounds that should not be recognized. Since the physical properties of the material

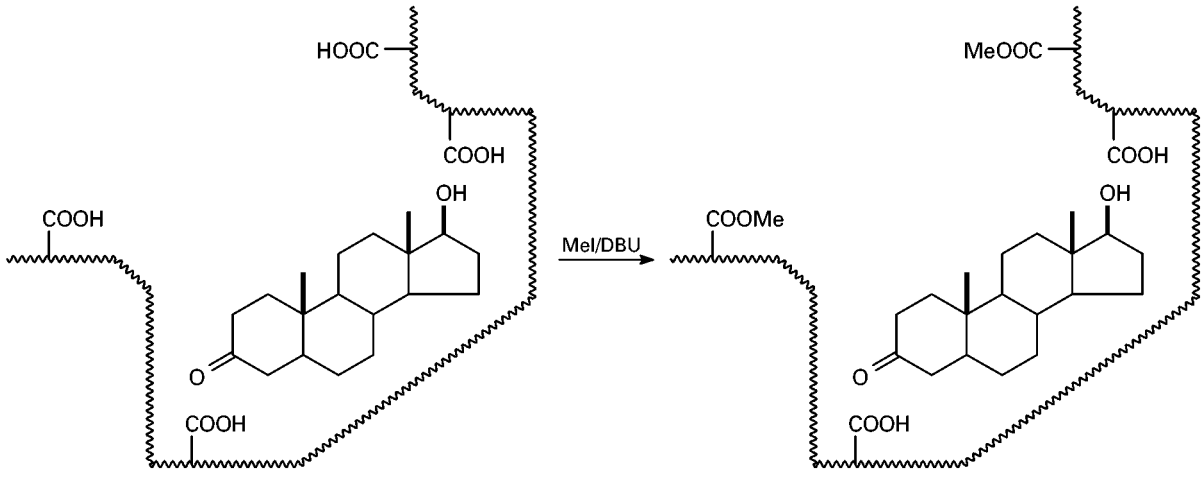

Figure 1 Reduction of nonspecific binding by chemical blocking.

will inevitably change upon imprinting, a change in retention will also be found for compounds not selectively recognized by the material. For example, if a material is prepared using methacrylic acid as functional monomer, the resulting imprinted material may show more pronounced retention than its nonimprinted counterpart. This phenomenon can in part be attributed to the formation of carboxylate dimers in the blank material, thus displaying fewer carboxylate groups free to interact with the analytes. On the other hand, in the imprinted material the carboxylate groups take part in the recognition process and are more exposed to the analytes. Thus, in order to estimate the selectivity of imprinted materials, an estimate of the behaviour of both imprinted and nonimprinted matrices needs to be made. For a full investigation, the behaviour of two or more imprinted matrices is also a very useful comparison. The nonimprinted matrix will address nonselective contributions for a chosen eluent. A set of imprinted matrices will provide information on both the selectivity accomplished and the physical change introduced by the imprinting process. Of course, when chiral separations are targeted, the chiral discrimination in itself is proof of the accomplished selectivity, since a nonimprinted matrix will normally never show enantioselectivity (unless chiral building blocks are used).

Selectivity

As mentioned above, the selectivities that can be accomplished with molecularly imprinted materials are often on a par with natural binders such as antibodies and biological receptors. In most cases, however, this can only be achieved by careful consideration of the system chosen, with respect to the ligand and the building block functionalities. Although much of the beauty and attraction of the entire concept lies in its seemingly compliant facility, design is none the less imperative for the outcome of an efficient imprinting process. It is only after judicious control of all system parameters that highly selective materials can be produced. Rational design is thus involved in using interactions as specific as possible from the very start. Most of the bond types used are chosen in order to acquire a desired selectivity directly from the resulting interaction with its corresponding counterpart, and this selectivity can subsequently be further accentuated by the imprinting process.

Molecular Imprinting Protocols

The characteristics of the imprinting process depend very much on the strength of the bonds used between the ligand and the functional building blocks, ranging from strong, albeit reversible, covalent bonds to weak, noncovalent interactions. What can be gained from an increased strength between the ligand and the functional building blocks during the imprinting process is often a drawback during the utilization of the resulting materials, and vice versa if noncovalent bond types are used. If too strong bonds are used, such as carboxylic amides and esters, the ease with which the extraction/rebinding process can procure is strongly limited, requiring exceedingly harsh conditions. Thus, weaker bond types have to be employed (**Figure 2**). Amongst reversible covalent bond types that have been used are boronic esters, imines and disulfides, all easily reversible under reasonably mild conditions. However, only boronic acid esters have been successfully used in chromatographic systems, due to their faster exchange kinetics, which is a reflection of their very low bond strength in aqueous environments ($\Delta G \sim 12\ \mathrm{kJ\ mol^{-1}}$ for the interaction between phenylboronic acid and glucose).

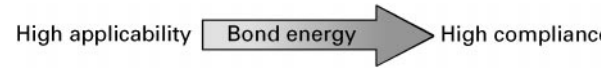

Figure 2 The higher the bond energy used for point interactions, the higher the compliance of the positioning of the groups in the site, but the lower the applicability of the concept. For this reason, most protocols are based on noncovalent systems.

The special characteristics of metal coordination make these very useful for molecular imprinting. The binding strengths lie between covalent and noncovalent, and can be easily varied by exchange of the metal ion used. The bond energy for a commonly used building block, iminodiacetate (IDA) complexed with Cu^{2+} and imidazole, amounts to $\sim 20\ \mathrm{kJ\ mol^{-1}}$ at room temperature in aqueous solution.

Although the use of reversible covalent bonds, as well as metal coordination, meets the prerequisite of strong interactions prior to fixation, these systems suffer from one serious drawback – only a limited number of ligands can be targeted using these bond types. Noncovalent interactions on the other hand permit a broader utility range that can be covered, largely making them more advantageous. This is in accordance with biological systems, where molecular complexes are often formed by a plethora of noncovalent interactions such as hydrogen bonds and ion pairing. Although these interactions, when considered individually, are weak compared to covalent bonds, the concerted action of several of these bond types often leads to complexes with very high stability. The high degree of specificity that can be achieved, in combination with the dynamic properties of the interactions, makes these bond types a prerequisite for many biological processes, and has also

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been the preferred choice in many imprinting protocols.

Attempts to overcome the drawbacks from the respective protocols, but still be able to benefit from their advantages, can be made by combining the two extremes. Thus, protocols based on covalent binding during the fixation process, but switching to noncovalent interactions afterwards, have been designed, for example, successful protocols based on cleavage of esters, carbonates and amides.

Origin of Selectivity

The complex, normally macromolecular nature of molecularly imprinted materials has precluded a full understanding of the recognition mechanism between the analyte and the matrix. Physical methods, such as solid-state nuclear magnetic resonance, Fourier transform infrared, atomic force microscopy (AFM) and electron spin resonance (ESR), have revealed some of the characteristics of the materials, but a more detailed account of the site architecture remains to be resolved. In contrast, the complexes between the functional building blocks and the print species, formed in solution prior to any imprinting process, can be studied more easily. Obviously, the use of covalent interactions, and often metal coordination interactions, results in structures that can be well characterized by common physical organic methods. In noncovalent systems the situation is more complex, but the use of solution-phase methods has allowed a picture of the interactions to be drawn. Although imprinting of these complexes/adducts may change their structures considerably, the information that can be retrieved from such studies is nevertheless valuable.

The recognition of events taking place in encounters between ligands and receptors is highly dependent on the additive effect of a number of binding forces. An optimal combination of the potential binding forces may lead to strong binding. Thus, in order to achieve proficient complexation between the host molecule and the guest species, several factors such as shape complementarity, functional complementarity and contributions from the surrounding pool of solvent have to be considered.

In all imprinting protocols, be they based upon covalent or noncovalent interactions, the most important factor governing the substrate selectivity is the quality and number of point interactions used to form bonds in solution prior to fixation, gelation or polymerization. In self-assembly systems using noncovalent interactions, the main factors responsible for selective rebinding of ligands to the imprinted sites have been shown to be strong noncovalent bonds such as charge–charge interactions and hydrogen bonding. Other types of interactions, such as π–π stacking and dipole interactions, may take part as well, but normally to a lesser extent. The three-dimensional arrangement of these interaction points, a reflection of the solution-phase situation set in place by the polymerization step, leads to an inherent specificity of the formed sites. Likewise, in metal coordination systems, the major points of interaction occur between the immobilized metal ions and the coordination sites of the analyte, and the reversible covalent interactions between the functional moieties of the matrix and their counterparts of the analyte make up the major interaction in covalent systems.

In addition to these point interactions, the architecture of the surrounding matrix determines the steric limits of the site, thus providing a more or less efficient van der Waals surface to the analyte. Although the arrangement of the functional moieties in the matrix accounts for most of the selectivity, the shape of the site also plays an important role. The configuration of the surrounding matrix backbone, as cast in place around the print molecule, contributes to the overall ligand specificity (**Figure 3**). This effect is sometimes less pronounced and substantial freedom in ligand structure can be observed, but often the effect is considerable and minute differences in size can be distinguished. In several cases, the position of a single methyl group is crucial for recognition. In this perspective, the shape effect of the site is particularly pronounced for parts of an imprinted molecule in close proximity to the point interactions. For structural features of the analyte more distal from such bonds, the shape is less important.

Forms of Selectivity

The topic of chemical selectivity can be categorized into three main groups:

1. chemoselectivity, i.e. differentiation among various functional groups in a polyfunctional molecule

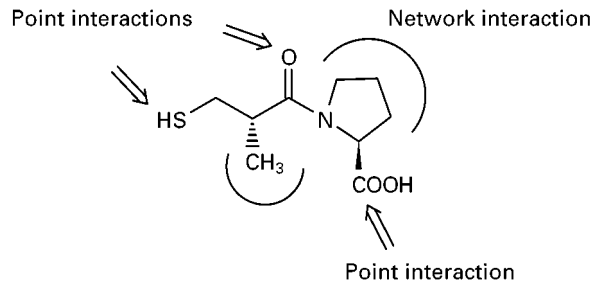

Figure 3 Schematic representation of interactions between a chosen ligand (here captopril) and a molecularly imprinted material. In addition to possible point interactions, whether covalent (disulfide, acetal, ester) or noncovalent (hydrogen bonds, coulombic electrostatic), the backbone of the imprinted network may also contribute to overall selectivity.

2. regioselectivity, referring to orientational control of the interaction
3. stereoselectivity, specifying the control of stereochemistry in the recognition site

Sometimes the last group is further subdivided with reference to the control of relative stereochemistry (diastereoselectivity) and absolute stereochemistry (enantioselectivity). A further physical type of selectivity is the discrimination of molecular size – an issue that may be of substantial importance when dealing with macromolecules and solid matrices.

Chemoselectivity In imprinting protocols based upon reversible covalent bonds, such as boronate esters, disulfides and imines, the chemoselectivity is principally a consequence of the properties of the covalent bond type chosen. Thus, for boronate esters, *vic*-diols are preferred as they give more stable esters than, e.g., mono-alcohols or *gem*-diols, and disulfides are formed exclusively from thiols. Other bond types of a slightly more general character, e.g. acetals, selective for carbonyl groups/alcohols, and imines, selective for carbonyl/amino moieties, are however potentially useful in recognizing a wider variety of ligands.

In metal coordination systems, the coordination properties of the ligand-metal ion pair govern most of the chemoselectivity that can be expected. This programmed selectivity can easily be changed, simply by changing the metal ion. If such a molecularly imprinted material is charged with different metal ions, the coordination properties can be fine-tuned. Such a bait-and-switch strategy has been used for changing the binding strength from high affinity during the imprinting process to low affinity in the rebinding/separation process. For example, Cu(II) can been changed for Zn(II), since the latter displays a weaker affinity for imidazole groups. When the ion itself is the target species (ligand), coordinating building blocks are normally chosen to occupy less than or equal to half of the coordinating bonds of the ion, allowing for adaptation during the imprinting process (**Figure 4**). The ionic recognition that can be achieved with such systems is often remarkable, and systems that are able selectively to distinguish small differences in ionic size and coordination pattern have been designed.

In noncovalent systems, the chemoselectivity is much less obvious in the design of the interactions. If a print molecule contains charges or hydrogen-bonding moieties, and functional monomers are chosen accordingly, the inherent chemoselectivity in the complexes formed is less strong, and other ligands can compete for the interactions as well. In addition, the functional building blocks, as well as the ligands, may self-interact to some extent. Nevertheless, a dramatic chemoselectivity can be accomplished when the strength of the noncovalent interaction is altered, for example, by introducing substituents which change the electronic density of a compound, or substituting a hydrogen taking part in a hydrogen bond. For example, caffeine (**1b**) binds very poorly to a molecularly imprinted material prepared against theophylline (**1a**), only differing in the exchange of a hydrogen for a methyl group, and the exchange of a hydroxyl group for a keto group in cortisol/cortisone (**2a**/**2b**) results in a remarkable loss in binding (**Figure 5**).

More elaborate systems, which can recognize certain motives in a given ligand, can however be designed. For example (**Figure 6**), the barbiturate structure (**3**), displaying two acceptor–donor–acceptor hydrogen bonding motifs, can be very selectively recognized in solution by a corresponding amidopyridine (**4**) displaying a donor–acceptor–donor counterpart. In comparison with monovalent functional mono-

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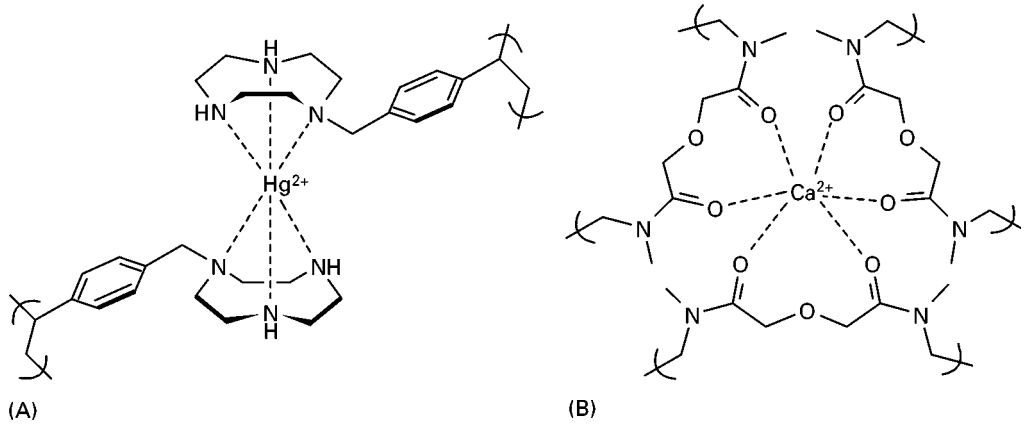

Figure 4 Metal ions can be very selectively recognized by imprinted matrices. For example, Hg(II) can be distinguished from Cd(II), Pb(II) and Cu(II) by the imprinted structure (A). Similarly, material (B) could selectively separate Ca(II) and Mg(II).

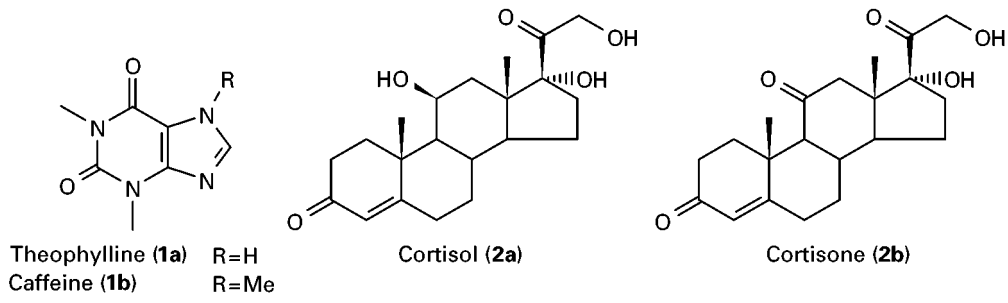

Figure 5 Examples of high chemoselectivity in noncovalent systems. Caffeine (**1b**) and theophylline (**1a**) differ only in one methyl group. Nevertheless, caffeine does not bind to matrices prepared against theophylline. Similarly, cortisone (**2b**) binds poorly to an anticortisol (**2a**) polymer.

mers, a high chemoselectivity can be designed for a chosen structure prior to the imprinting process. On the other hand, if a carefully designed multipoint system does not allow for dynamic adjustment, not much is gained from the imprinting process. In this example, the general structure (**3**) is well recognized, irrespective of *R* and *R′*.

In all these cases, however, the chemoselectivity that is more or less rationally designed upon examination of the target print species can be radically enhanced by the imprinting process. This is particularly the case when dealing with less specific noncovalent interactions. The positioning of the functional groups in the three-dimensional network of the matrix, together with the formation of the site or imprint, often results in a tremendous increase in specificity.

Regioselectivity Regioselectivity can often be nicely demonstrated by molecularly imprinted materials using all types of imprinting protocols. In this case, the imprinting process *per se*, rather than the bond type, endows high regioselectivity. It is mainly due to proper three-dimensional (or two-dimensional) positioning of the functional building blocks in the finally produced matrix that high selectivity can be accomplished. Sometimes, however, the selectivity is also a consequence of the architecture of the surrounding backbone of the imprinted matrix in addition to organized point interactions. Obviously, this works concertedly, such that the build-up of the matrix backbone may force the originally noninteracting parts of the functional building blocks to accommodate a position, resulting in steric hindrance of different analytes.

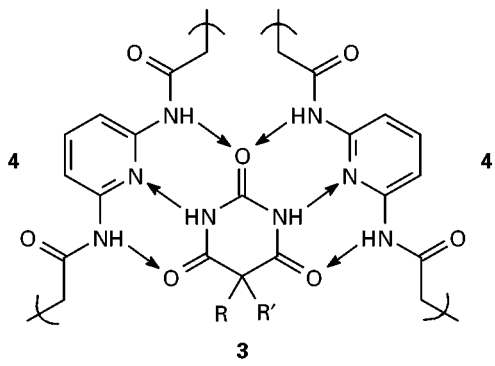

Figure 6 Multiple hydrogen bonding between cyclobarbital (**3**) and *bis*-amidopyridines (**4**).

A number of systems have been designed where *bis*-functional compounds displaying a difference in distance between the point interactions can be efficiently distinguished by the produced matrix. For example, a range of either *bis*-aldehydes (**5a**/**5b**) or diketones (**6a**/**6b**) could be selectively discriminated by matrices produced using imine- and acetal-formations, respectively (**Figure 7**).

Another example is the imprinting of *bis*-imidazole structures using Cu(II) coordination. A selectivity between the structures could be recorded, largely dependent upon the positioning of the Cu ions in the material. In these examples, the distance between the point interactions can be considered as being of major importance for recognition.

Similar studies have been pursued using noncovalent interactions, e.g. the interactions of *bis*-pyridyl and *bis*-aniline compounds with carboxylic acids, which show high distance selectivities also.

Although investigations of such *bis*-compounds can be considered as interesting model studies, proving the utility of the imprinting concept, many examples of compounds of more practical interest, e.g. food additives or drugs, have been examined. Steroids, for instance, are a class of compounds that lends itself to being both interesting model systems as well as commercially important, and several impressive steroid separations have been demonstrated. For example, β-11-OH-progesterone (**7a**) can be most selectively separated from β-17-OH-progesterone (**7b**) (**Figure 8**), in part due to the reasonably rigid steroid framework, which helps to lower the entropy loss upon steroid binding compared to a more flexible molecule.

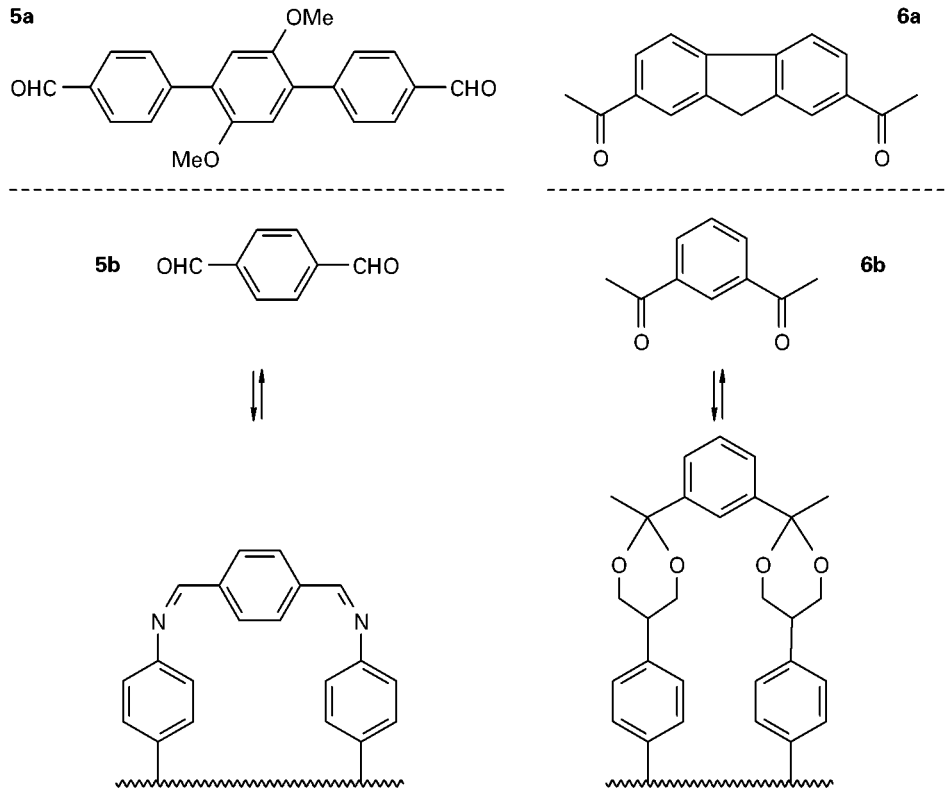

Figure 7 Distance selectivity in reversible covalent systems. *Bis*-aldehydes **5a**/**5b**, and *bis*-ketones **6a**/**6b** could be distinguished by matrices prepared against one of the structures using imine and ketal formation, respectively.

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Stereoselectivity The physical properties of enantiomers, i.e. identical properties in a symmetrical environment, render them ideal substrates for the study of imprinting specificity. This is one of the reasons why this aspect has been the most extensively studied in MIT in general, and in MIC in particular. Indeed, this quality offers the clearest evidence for the outcome of a successful imprinting process. Obviously, if chiral discrimination can be introduced in a matrix through an imprinting process, based upon exclusively achiral building blocks, an indisputable demonstration of the concept is obtained.

A large number of investigations concerning chiral separations by molecularly imprinted materials have been performed, and hardly any type of compound has been neglected from examination. Thus, compounds such as amino acids, carbohydrates, nucleic acids and pharmaceuticals have all been applied in various imprinting protocols. The stereoselectivity that can be achieved is also quite extraordinary, and differences in binding of single hydroxyl groups (*R*- and *S*-timolol), and even single methyl groups can sometimes be observed (*R*- and *S*-naproxen). The interested reader can find a more detailed overview of chiral separations using molecularly imprinted materials elsewhere in this Encyclopedia (Kempe M, Chiral Separation: Molecular Imprints as Stationary Phases).

Conclusions and Future Prospects

MIT is a technique that has substantially improved over the last few years. Many of the drawbacks initially encountered have gradually been overcome, and intense research is ongoing to resolve some of the remaining challenges. As has been pointed out, the technique is already competent at recognizing very small structural differences, and the ultimate venture that prevails in this respect is the separation of isotopes. Another task that needs further attention, although some steps have already been taken in this direction, is the selective recognition/separation of biological macromolecules, and even whole cells.

For affinity separation, however, high affinities may not always be the ultimate goal. High affinities sometimes lead to situations where elution from the affinity matrix requires harsh condition. For this reason, a material providing a lower affinity can sometimes be advantageous, and the process of binding/elution can be accomplished using mild conditions. Likewise, a high selectivity is not always a goal in itself, and materials with lower selectivity

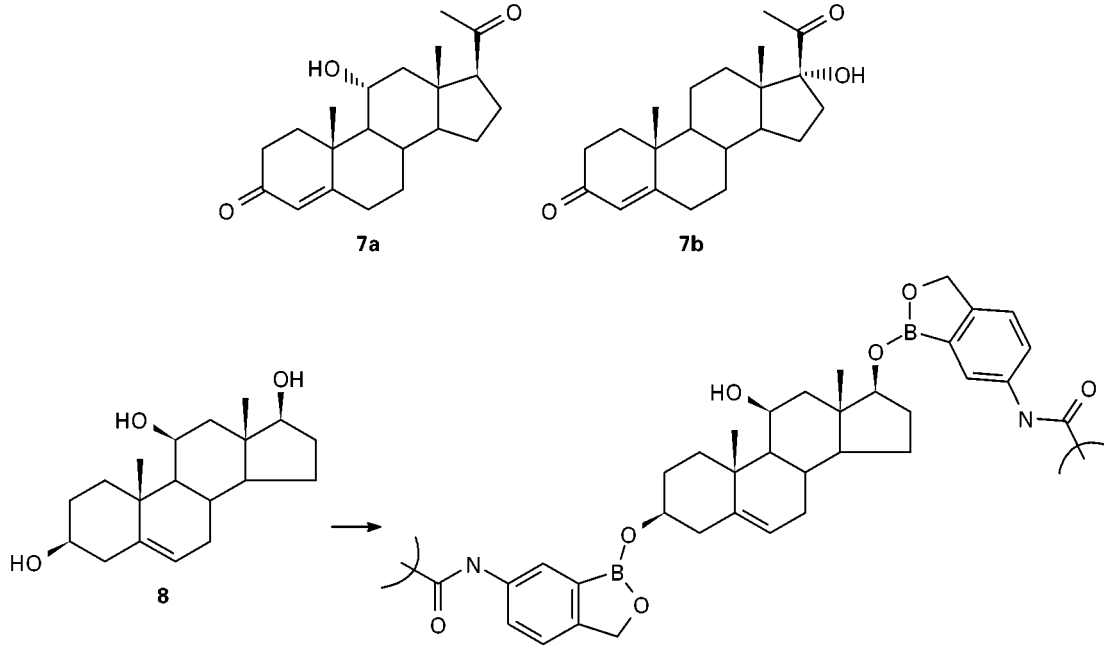

Figure 8 Examples of the high regioselectivity that can be achieved by matrices produced against steroids. β-11-OH-progesterone (**7a**) and β-17-OH-progesterone (**7b**) were efficiently separated by a molecularly imprinted chromatographic stationary phase. The androstene-triol (**8**) could be selectively acetylated in the 11-position upon protection/binding to the matrix.

can also be preferable. This is the case when performing separations of groups of molecules rather than a single species, such as the recognition of the β-lactam group as a whole, rather than individual penicillins. Using materials that are apt at recognizing a 'molecular chord', rather than single 'molecular notes', can sometimes be beneficial.

See also: **I/Affinity Separation. II/Affinity Separation:** Immunoaffinity Chromatography; Imprint Polymers. **III/Chiral Separations:** Molecular Imprints as Stationary Phases. **Immunoaffinity Extraction. Molecular Imprints for Solid-Phase Extraction. Selectivity of Imprinted Polymers: Affinity Separation.**

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